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(54) Title: STIMULATION OF HEMATOPOIESIS BY EX VIVO ACTIVATED IMMUNE CELLS

(57) Abstract: A protocol of activating and administering human blood cells so that bone marrow histology and/or blood cell counts of patients suffering from aplastic anemia approach normal. The protocol includes culturing the blood cells in the presence of a cytokine and an ionophore.

STIMULATION OF HEMATOPOIESIS BY EX VIVO ACTIVATED IMMUNE CELLS

Background of the Invention

5 1. Field of the Invention

This invention relates to therapies for aplastic anemia, anemia and thrombocytopenic purpura. In particular, this invention relates to ex vivo activated immune cells as therapies for aplastic anemia, anemia and thrombocytopenic purpura. Furthermore, the invention relates to approaches to activate cells and corresponding cell
10 culture approaches.

2. Background

Aplastic anemia is a disease characterized by ineffective hematopoiesis. Patients have varying degrees of abnormalities in production of all blood cell types. Although in
15 most cases, the cause of the disease is unknown, radiation, benzene-based compounds, viruses (e.g., hepatitis), environmental toxins, and over the counter and prescription medications have been suspected to damage bone marrow, thereby leading to apoptosis of marrow stem cells. Regardless of the underlying causes, patients show similar clinical manifestations and disease progression courses. Aplastic anemia affects primarily young
20 man and older persons of both the genders. Annually, two to six per million worldwide develop this disorder, with a prevalence of incidences in the Orient as compared to Europe or the United States. Several causal phenomena are hypothesized for aplastic anemia: congenital, pregnancy, viral, and drugs and chemicals.

The most frequently cited causal agent of aplastic anemia is drugs or chemical
25 exposure. Some agents, such as chloramphenicol, benzene, ionizing radiation, and antineoplastic agents, cause an aplasia that is dose-related in severity from person-to-person. In these cases, marrow recovery usually occurs after withdrawal of the causal agent. Other agents, including pesticides and some anticonvulsants and antimicrobials, cause a reaction which is not dose-related and, therefore, cannot be predicted with
30 hematological monitoring during administration. During administration of drugs, aplasias may occur even after cessation of drug therapy. In contrast to patients with idiopathic aplastic anemia, those with drug or toxin exposure exhibit similar clinical and demographic characteristics, have a similar prognosis, and a more-or-less uniform response to therapy.

In the case of benzene-induced aplastic anemia, mild to moderate disease symptoms usually disappear after patients cease being exposed to benzene. However, for patients with severe bone marrow failure or who continually need blood transfusions, effective and safe treatment has not often been heretofore available. To date, bone marrow transplantation is the only known cure.

Mild aplastic patients are often treated with as little therapy as possible. The rationale for minimum treatment for mildly aplastic patients is to remove the causal agent, thereby enabling spontaneous recovery. In young patients with severe anemia, bone marrow transplantation with an HLA-matched donor is the treatment of choice. Bone marrow transplantation effects complete remission in nearly 80% of cases. However, survival decreases to 10-20% when the donor and recipient are mismatched at two or more loci. Complications associated with transplantation include graft rejection, acute or chronic graft-versus-host disease, infection, and other miscellaneous organ specific damage. Marrow transplant recipients also have an increased long-term risk for developing subsequent solid tumors.

Summary of the Invention

The present inventor has investigated using cultured (activated) blood cells for treating patients with blood deficiencies, such as anemia, aplastic anemia and/or thrombocytopenic purpura. In one embodiment of the invention, a quantity of blood cells effective to treat blood deficiencies when injected into a patient is provided. The quantity of blood cells may be cultured in the presence of a cytokine and an ionophore. The cytokine and ionophore may be present in effective concentrations. The cytokine may comprise interleukin-2 and macrophage-colony stimulating factor. The ionophore may comprise A23187.

In another embodiment, the present invention provides a process of treating blood deficiencies in a patient, the treatment comprising administering ex vivo cultured blood cells to the patient. A therapeutically effective amount of blood cells may be administered to the patient. The blood cells may be autologous to the patient, allogeneic to the patient, or from an immunologically acceptable owner. The blood cells may further be cultured in the presence of a cytokine and an ionophore. The cytokine and ionophore may be present in effective amounts.

In yet another embodiment, the present invention provides a method of culturing blood cells, the method comprising culturing the blood cells in the presence of a cytokine

and an ionophore. The blood cells may be cultured in the presence, for example, of effective amounts of the cytokine and ionophore; may be cultured in a medium which may or may not comprise mammalian serum; may be cultured for a period, for example, between about 2 and 200 hours or longer; and may be cultured at a temperature, for example, between about 30 and 42 degrees C. The cytokine may include interleukin-2 and/or granulocyte macrophage-colony stimulating factor. The ionophore may include A23187.

Brief Description of the Drawings

Fig. 1 shows low-power views of H&E stained bone marrow biopsies from three patients responsive to the present therapy. The views labeled as A, C and E are marrows from patients before treatment. In these views, early empty and impaired marrows implicate severe aplastic anemia. In the views denoted as B, D and F, the marrows are from the same patients after treatment. These marrows show much improved distribution and cellularity.

Fig. 2 is a plot of platelet count in platelets per cubic millimeter as a function of days after starting treatment with activated cells for a child pateint with platelet deficiency.

Detailed Description

The present invention includes a therapy of administering a therapeutically effect amount of ex vivo cultured blood cells to patients afflicted with blood deficiencies, such as anemia, aplastic anemia and thrombocytopenic purpura. The term "therapeutically effective amount" is intended to include a sufficient quantity of the present activated blood cells to effect a statistically significant increase in blood cell counts when administered to a patient with blood deficiencies, i.e., a significantly low concentration of a natural blood component, such as red blood cells, white blood cells, platelets and other factors produced by the bone marrow and cells generated from the bone marrow. The cultured blood cells may be either from the patient or from an immunologically acceptable donor. One protocol for activating blood cells via ex vivo culture includes obtaining a blood sample (e.g., 10-100 ml) from the patient, or an immunologically acceptable donor, separating blood cells from the blood sample, and culturing the separated blood cells. An "immunologically acceptable donor" is a person having tissues, to include blood cells, that do not have medically unacceptable levels of recipient reactions (e.g., hemolytic anemia, heart failure, renal failure). The blood cells may be separated from blood sera by

protocols such as by centrifugation. The separated blood cells are then cultured under sterile conditions in a medium with one or more of a cytokine (to include cell stimulating factors) and an ionophore. The separated blood cells may be cultured in the media as specified above, for example, for periods between of greater than about 1 hour, in other
5 embodiments between about 10 and 200 hours, between about 20 and 80 hours, or between about 30 and 60 hours and at a temperature, for example, between about 30 and 42 degrees C, in other embodiments between about 32 and 40 degrees C, or between about 37 and 38 degrees C or any range subsumed therein. A person of ordinary skill in the art will recognize that other ranges of periods and temperatures within these explicit ranges
10 are contemplated, and are within the present disclosure.

Blood deficiencies can be treated by the approaches described herein. In general, the blood deficiencies involve a reduced concentration of blood components that originate from the bone marrow or from products, such as specific cell types, from the bone marrow. Blood deficiencies include, for example, anemia, aplastic anemia
15 and thrombocytopenic purpura. Anemia can be considered broadly as a deficiency of a blood component or, in some contexts, as a deficiency of red blood cells. Aplastic anemia is a deficiency of peripheral blood elements. Thrombocytopenic purpura, such as idiopathic thrombocytopenic purpura, involves a deficiency in platelet number. As a specific example, the discussion below describes aplastic anemia in some detail, although
20 the treatment methods can be applicable more broadly.

After being cultured, the activated blood cells may be washed (e.g., twice with sterile saline solution). Therapeutically effective amounts of the activated blood cells are then administered to patients. One acceptable method of administering the activated blood cells is intravenously. While the activated cells may be administered in a single dose,
25 portions of the activated blood cells may also be administered over a period of time. For example, doses of the present activated blood cells may be administered to patients once per week for a period of four weeks. However doses of the present activated blood cells may be administered to patients at intervals of, for example, one-half week, ten days, 14 days, 21 days, other intermediate periods, or other effective periods. Moreover, the
30 intervals may vary during the course of the treatment. For example, initially blood cell doses may be administered at daily, twice a week, weekly, and/or bi-weekly intervals. The dosages can be, for example, between about 1×10^5 to about 2×10^8 cells per treatment, which may depend on the patient's age and condition. The total time required for treatment (e.g., administering the present activated blood cells) may depend on the

amount of activated blood cells available and patient response. Patient response can be measured, for example, in terms of return to normal blood cell counts and/or marrow histology as well as an overall improvement in health. Obviously, blood samples can be drawn from patients repeatedly during or after the initial treatment period so that additional activated blood cells can be obtained for further treatments. Furthermore, activated blood cells from an immunologically acceptable donor can be administered initially or administered for the entire duration of the treatment. Alternatively, blood cells from the patient, activated by the present protocol, may be administered after blood cells from an immunologically acceptable donor are initially administered.

The most current definition of severe aplastic anemia is marked pancytopenia with at least two of the following: 1) granulocytes less than 500/microliter, 2) platelets less than 20,000/microliter, 3) anemia with corrected reticulocyte count less than 1%, plus markedly hypoplastic marrow depleted of hematopoietic cells. Moderate aplastic anemia generally involves a hypocellular bone marrow and cytopenia in at least two cell lines not in the severe range. Onset is insidious and the initial complaint may be progressive fatigue and weakness due to the anemia, followed in some cases by hemorrhage. The hemorrhage is usually from the skin and mucosal linings, due to thrombocytopenia. Infection is rare despite the severe neutropenia. Physical examination reveals pallor and possibly bruising or petechiae. Aplastic anemia patients exhibit no lymphadenopathy or splenomegaly. Fever may or may not be present. Peripheral blood assays show pancytopenia. The presence of immature red and white blood cells strongly argues against aplastic anemia.

Red blood cells may be mildly macrocytic due to increased erythropoietic stress and they usually are normocytic and normochromic. The corrected reticulocyte count is very low or zero, indicating a lack of erythropoiesis. Bleeding time may be prolonged even with normal coagulation parameters. Patients have an increased serum iron and a normal transferrin, resulting in an elevated transferrin saturation. Plasma iron clearance is decreased due to a reduction in erythropoiesis. Bone marrow aspirate may be dry. But a biopsy can show severe hypocellular or aplastic marrow with fatty replacement. Because there have been cases in which the initial marrow biopsy exhibited hypercellularity, more than one biopsy may be necessary for accurate diagnosis. A severe depression can be noted in all hematopoietic progenitor cells, including myeloid, erythroid, pluripotent cell lines, and megakaryocytes. Diagnosis generally is based on finding the classic triad of anemia, neutropenia, and thrombocytopenia in both blood and bone marrow specimens. X-rays may be needed to rule out bone lesions or neoplastic infiltrates. Magnetic

resonance imaging has been useful in clearly defining hypoplastic marrow. Since the diagnosis is one of exclusion, all other causes of pancytopenia and other lab findings are usually ruled out before aplastic anemia can be diagnosed.

The basic defect in aplastic anemia is failure of production of all cell lines.
5 Possible mechanisms of the pathogenesis of aplastic anemia include 1) defective or absent hematopoietic stem cells, 2) abnormal bone marrow microenvironment, 3) abnormal regulatory cells, and 4) suppression of hematopoiesis by immunologic cells.

While the pathophysiology of the disease is not yet completely clear, (Young et al., The pathophysiology of acquired aplastic anemia, N. Engl. J. Med. 1997; 336(19): 1365-1372 and Young et al., The treatment of severe acquired aplastic anemia, Blood. 1995; 10 85(12): 3367-3377) there is evidence to support the theory that aplastic anemia is an immune-mediated disease. Bone marrow transplantation and immunosuppressive therapy using combined antilymphocyte globulin and cyclosporine have been used for treatment (Rosenfeld et al., Intensive immunosuppression with antithymocyte globulin and 15 cyclosporine as treatment for severe aplastic anemia, Blood 1995; 85(11): 3058-3065 and Halperin et al., Severe acquired aplastic anemia in children: 11-year experience with bone marrow transplantation and immunosuppressive therapy, Am. J. Pediatr. Hematol. Oncol. 1989; 11(3): 304-309). However, the therapy of immune suppression often has undesirable and severe side effects. Moreover, hematopoietic growth factors such as 20 granulocyte colony-stimulating factor (Kojima et al., Treatment of aplastic anemia in children with recombinant human granulocyte-colony stimulating factor, Blood 1991; 77(5): 937-941 and Sonoda et al., Multilineage response in aplastic anemia patients following long-term administration of filgrastim (recombinant human granulocyte colony stimulating factor), Stem Cells 1993; 11: 543-554), granulocyte macrophage colony-stimulating factor (Champlin et al., Treatment of refractory aplastic anemia with 25 recombinant human granulocyte-macrophage-colony-stimulating factor, Blood 1989; 73(3): 694-699 and Guinan et al., A phase I/II trial of recombinant granulocyte-macrophage colony-stimulating factor for children with aplastic anemia, Blood 1990; 76(6): 1077-1082), and Interleukin-3 (Ganser et al., Effect of recombinant human interleukin-3 in patients with normal hematopoiesis and in patients with bone marrow 30 failure, Blood 1990; 76(4): 666-676 and Nimer et al., A phase I/II study of interleukin-3 in patients with aplastic anemia and myelodysplasia, Exp. Hematol. 1994; 22: 875-880) have provided only limited and transient effects.

Many patients respond to immunosuppressive therapy and there are abnormal

levels of various immune molecules in aplastic patients. For instance, Interleukin-1, produced by macrophages, natural killer cells, B lymphocytes, and endothelial cells, plays a central role in both immune responses and regulation of hematopoiesis by inducing the release of erythroid and multipotent colony-stimulating factors from marrow stromal cells, regulating early progenitor cells and stimulating stem cell recovery following induced myelosuppression. Immune dysregulation in aplastic anemia consists of decreased natural killer cell activity, increased numbers of activated T suppressor cells and abnormal production of Interleukin-2 and gamma-Interferon.

Natural killer cells are large granular lymphocytes which lyse tumor cells or virus-infected target cells upon direct contact. Natural killer cells also produce gamma-interferon, Interleukin-2, and induces colony-stimulating activity. These cells may inhibit myeloid and erythroid colony formation under certain conditions. For instance, when exogenous growth factors are absent from a culture, natural killer cells normally produce cytokines and support hematopoiesis. However, optimal conditions induce natural killer cells to inhibit hematopoiesis. Natural killer cell activity in aplastic anemia patients returns to normal after hematopoietic recovery.

Gamma-Interferon is produced by activated lymphocytes and suppresses hematopoiesis. Although aplastic patients show an overproduction of gamma-Interferon, levels of gamma-Interferon decrease in response to immunosuppression. Interferons are potent inhibitors of hematopoietic colony formation - both through direct action on progenitor cells and indirect effects via accessory immune system cells.

Tumor necrosis factor-alpha is another cytokine which is in excess in aplastic anemia. It functions to inhibit colony growth of the normal hematologic progenitors. High tumor necrosis factor-alpha values correlate with decreased platelet, hemoglobin, and leukocyte counts. Tumor necrosis factor-alpha and gamma-Interferon may act synergistically to suppress hematopoiesis.

Aplastic anemia patients produce gamma-Interferon and tumor necrosis factor-alpha in excess, show an inverted helper:suppressor T cell ratio, and have predominantly T suppressor cells in the bone marrow. These cells may mediate suppression of hematopoiesis via cytokine production. The bone marrow also has a higher proportion of cytotoxic T cells than peripheral blood. The clinical relevance of immune dysfunction is suggested by a decrease in activated lymphocytes following successful immunosuppressive therapy.

Mechanisms for acquired aplastic anemia in general, and mechanisms for benzene-

induced aplastic anemia in particular, are not well understood. Nonetheless, both types of aplastic anemia share considerable similarities with respect to pathophysiology and clinical manifestations. There are presently two hypotheses to explain the mechanism of aplastic anemia, direct damage and immune-mediated. Both hypotheses are supported by data from experimental and clinical studies. Direct damage to bone marrow cells is thought to be responsible for temporary and reversible bone marrow failure following cytotoxic chemotherapy and radiotherapy. Immune-mediated bone marrow failure is more difficult to cure. In the case of benzene-induced aplastic anemia, the disease seems to be associated with both mechanisms. Evidence of direct damage to bone marrow cells is supported by the studies indicating that benzene is involved in inhibiting a number of biochemical processes of bone marrow cells. Specifically, benzene has been shown to damage stromal macrophages in bone marrow, thereby leading to deficient interleukin-1 production (Niculescu et al., Inhibition of the conversion of pre-interleukins-1[alpha] and 1[beta] to mature cytokines by p-benzoquinone, a metabolite of benzene, *Chemico-Biological Interactions*; 1995; 98: 211-222 and Kalf et al., p-benzoquinone, a reactive metabolite of benzene, prevents the processing of pre-interleukins-1[alpha] and -1 [beta] to active cytokines by inhibition of the processing enzymes, calpain, and interleukin-1 [beta] converting enzyme, *Environmental Health Perspectives*; 1996; 104 (suppl. 6): 1251-1256). Interleukin-1 is considered important for growth and differentiation of stem cells (Bagby, G.C., Production of multi lineage growth factors by hematopoietic stromal cells: an intercellular regulatory network involving mononuclear phagocytes and interleukin-1, *Blood Cells* 1987; 13:147-159 and Fibbe et al., Human fibroblasts produce granulocyte-CSF, macrophage-CSF and granulocyte-macrophage-CSF following stimulation by interleukin-1 and poly(rl).poly(rC), *Blood* 1988; 72(3): 860-866). However, there has been no report of prolonged response to treatments of hematopoietic growth factors, including interleukin-1.

Medium.

Suitable media used in ex vivo activation provide essential nutrients for blood cells. These media generally comprise, for example, inorganic salts, amino acids, vitamins, and other compounds all in forms which can be directly utilized by blood cells. By way of illustration and not limitation, one suitable medium is RPMI 1640. However, other media, such as serum-free media AIM-V, will support blood cells in culture may be suitable as well. The medium may be supplemented with a mammalian serum, e.g., fetal

bovine serum at levels between about 0.1 and 50%, between about 1 and 40%, or between about 5% and 15%, of the medium, by weight. One suitable formulation of RPMI, designated as a modified RPMI 1640 and available under catalog number 30-2001 from American Type Culture Collection, has the following ingredients:

5		<u>Inorganic Salts</u>	<u>(g/liter)</u>
		Ca(NO ₃) ₂ · 4H ₂ O	0.10000
		MgSO ₄ (anhydrous)	0.04884
		KCl	0.40000
10		NaHCO ₃	1.50000
		NaCl	6.00000
		Na ₂ HPO ₄ (anhydrous)	0.80000
		<u>Amino Acids</u>	<u>(g/liter)</u>
15		L-Arginine (free base)	0.20000
		L-Asparagine · H ₂ O	0.05682
		L-Aspartic Acid	0.02000
		L-Cystine · 2HCl	0.06520
		L-Glutamic Acid	0.02000
20		L-Glutamine	0.30000
		Glycine	0.01000
		L-Histidine (free base)	0.01500
		Hydroxy-L-Proline	0.02000
		L-Isoleucine	0.05000
25		L-Leucine	0.05000
		L-Lysine · HCl	0.04000
		L-Methionine	0.01500
		L-Phenylalanine	0.01500
		L-Proline	0.02000
30		L-Serine	0.03000
		L-Threonine	0.02000
		L-Tryptophan	0.00500
		L-Tyrosine · 2Na · 2H ₂ O	0.02883
35		L-Valine	0.02000
		<u>Vitamins</u>	<u>(g/liter)</u>
		D-Biotin	0.00020
		Choline Chloride	0.00300
		Folic Acid	0.00100
40		myo-Inositol	0.03500
		Nicotinamide	0.00100
		p-Amino Benzoic Acid	0.00100
		D-Pantothenic Acid	0.00025
		(hemicalcium)	
45		Pyridoxine · HCl	0.00100
		Riboflavin	0.00020

Thiamine· HCl	0.00100
Vitamin B-12	0.000005

<u>Other</u>	<u>(g/liter)</u>
D-Glucose	4.50000
Glutathione (reduced)	0.00100
HEPES	2.38300
Phenol Red, Sodium Salt	0.00500
Sodium Pyruvate	0.11000

1. Cytokines. One or more cytokines may be used to activate blood cells when cultured in the presence thereof. Cytokines are small proteins (usually in the range of 5-20 kD) that are released by cells and have specific effects on cell-cell interaction, communication, and behavior of other cells. Usually included as cytokines, are interleukins, lymphokines and signaling molecules such as tumor necrosis factor (TNF) and interferons. While natural cytokines can be used, recombinant produced cytokines produced, for example, by established nucleic acid expression systems are also contemplated. As such, modified and mutated forms of natural cytokines that maintain function can also be used. Exemplary cytokines, which may be suitable for some embodiments of the present invention, include:

A. Interleukins. A variety of naturally occurring polypeptides that affect functions of specific cell types and are found in small quantities. They are secreted regulatory proteins produced by lymphocytes, monocytes and various other cells and are released by cells in response to antigenic and non-antigenic stimuli. The interleukins, of which there are 16 identified to date, modulate inflammation and immunity by regulating growth, mobility and differentiation of lymphoid and other cells. Interleukins may be present in concentrations between about 10 and 50,000 IU/ml, about 100 - 5,000 IU/ml, or about 100 - 1,000 IU/ml. Alternatively an effective concentration of interleukins may be present. An effective concentration of interleukins is any concentration at which blood cells are activated by the present protocol.

i. Interleukin-1 (IL-1). IL-1 is a soluble protein (17 kD: 152 amino acids) secreted by monocytes, macrophages or accessory cells involved in the activation of both T lymphocytes and B

lymphocytes and potentiates their response to antigens or mitogens. Biological effects of IL-1 include the ability to replace macrophage requirements for T-cell activation, as well as affecting a wide range of other cell types. At least two IL-1 genes are known and alpha and beta forms of IL-1 are recognized. IL-1 is released early in an immune system response by monocytes and macrophages. It stimulates T-cell proliferation and protein synthesis. Another effect of IL-1 is to cause fever.

ii. Interleukin-2 (IL-2). IL-2 is a hormone-like substance released by stimulated T lymphocytes. IL-2 causes activation and differentiation of other T lymphocytes independently of antigen. IL-2 stimulates the growth of certain disease-fighting blood cells in the immune system and is secreted by Th1 CD4 cells to stimulate CD8 cytotoxic T lymphocytes. IL-2 also increases the proliferation and maturation of CD4 cells themselves.

iii. Interleukin-3 (IL-3). IL-3 is a product of mitogen activated T-cells. IL-3 is a colony stimulating factor for bone marrow stem cells and mast cells. IL-3 is considered one of the hematopoietic colony stimulating factors.

iv. Interleukin-4 (IL-4). IL-4 is a soluble cytokine factor produced by activated T lymphocytes that promotes antibody production by causing proliferation and differentiation of B-cells. IL-4 induces the expression of class II major histocompatibility complex and fc receptors on B-cells. IL-4 also acts on T lymphocytes, mast cell lines, and several other hematopoietic lineage cells including granulocyte, megakaryocyte, and erythroid precursors, as well as macrophages.

v. Interleukin-5 (IL-5). IL-5 is a factor promoting eosinophil differentiation and activation in hematopoiesis. It also triggers activated B-cells for a terminal differentiation into Ig-secreting cells.

vi. Interleukin-6 (IL-6). IL-6 stimulates the growth and differentiation of human B-cells and is also a growth factor for hybridomas and plasmacytomas. It is produced by many different

cells including T-cells, monocytes, and fibroblasts. IL-6 is a single chain 25 kD cytokine originally described as a pre B-cell growth factor, now known to have effects on a number of other cells including T-cells which are also stimulated to proliferate.

5 vii. Interleukin-7 (IL-7). IL-7 is a hematopoietic growth factor that promotes growth of B-cell precursors and is also co-mitogenic with interleukin-2 for mature T-cell activation. IL-7 is produced by bone marrow stromal cells.

10 viii. Interleukin-8 (IL-8). IL-8 is a cytokine that activates neutrophils and attracts neutrophils and T lymphocytes. IL-8 is released by several cell types including monocytes, macrophages, T lymphocytes, fibroblasts, endothelial cells, and keratinocytes by an inflammatory stimulus. IL-8 is a member of the beta-thromboglobulin superfamily and structurally related to platelet factor 4.

15 ix. Interleukin-9 (IL-9). IL-9 is a cytokine produced by T-cells, particularly when mitogen stimulated. IL-9 stimulates the proliferation of erythroid precursor cells (BFUE) and is thought to be a regulator of hematopoiesis. IL-9 may act synergistically with erythropoietin. The IL-9 receptor belongs to the hemopoietic receptor super family. IL-9 has been shown to enhance the growth of human mast cells and megakaryoblastic leukaemic cells as well as murine helper T-cell clones. IL-9 is a glycoprotein that is derived from T-cells and maps to human chromosome 5.

20 x. Interleukin-10 (IL-10). IL-10 is a factor produced by Th2 helper T-cells, some B-cells and LPS activated monocytes. It is a coregulator of mast cell growth.

25 xi. Interleukin-11 (IL-11). IL-11 is a pleiotropic cytokine, originally isolated from primate bone marrow stromal cell line, that has the ability to modulate antigen-specific antibody responses, potentiate megakaryocytes, and regulate bone marrow adipogenesis. IL-11 stimulates T-cell dependent B-cell maturation, megakaryopoiesis, and various stages of myeloid differentiation.

30 xii. Interleukin-12 (IL-12). IL-12 is a 75 kD heterodimeric

cytokine composed of disulfide-bonded 40 kD and 35 kD subunits that was originally identified by its ability to induce cytotoxic effector cells in synergy with less than optimal concentrations of interleukin-2. IL-12 is released by macrophages in response to infection and promotes the activation of cell-mediated immunity. Specifically, IL-12 triggers the maturation of Th1 CD4 cells, specific cytotoxic T lymphocyte responses, and an increase in the activity of NK cells. Consequently, IL-12 is the initiator of cell-mediated immunity. It enhances the lytic activity of NK cells, induces interferon production, stimulates the proliferation of activated T-cells and NK cells. Is secreted by human B lymphoblastoid cells (NC 37).

xiii. Interleukin-13 (IL-13). IL-13 is a T lymphocyte-derived cytokine that produces proliferation, immunoglobulin isotype switching, and immunoglobulin production by immature B-lymphocytes. IL-13 is produced by activated T-cells, inhibits IL-6 production by monocytes, and also inhibits the production of other pro-inflammatory cytokines such as TNF, IL-1, and IL-8. IL-13 stimulates B-cells. The gene for IL-13 is located on human chromosome 5q in a gene cluster that also has the IL-4 gene.

xiv. Interleukin-14 (IL-14). IL-14 is a cytokine that induces B-cell proliferation, inhibits immunoglobulin secretion, and selectively expands certain B-cell subpopulations.

xv. Interleukin-15 (IL-15). IL-15 is a cytokine that stimulates the proliferation of T lymphocytes and shares biological activities with IL-2. IL-15 also can induce B lymphocyte proliferation and differentiation.

xvi. Interleukin-16 (IL-16). IL-16 is a cytokine produced by activated T lymphocytes that stimulates the migration of CD4-positive lymphocytes and monocytes.

B. Lymphokines. A lymphokine is a substance produced by a leucocyte that acts upon another cell. Examples are interleukins, interferon alpha, lymphotoxin (tumor necrosis factor alpha), granulocyte monocyte colony stimulating factor (GM-CSF).

5 i. Interferons (IFN) are a family of glycoproteins human cells which normally have a role in fighting viral infections by preventing virus multiplication in cells. Interferons may be present in the same concentrations as interleukins. Alternatively, effective concentrations of interferons may be present. Effective concentrations of interferons are contemplated to include any concentration at which blood cells are activated by the present protocol. IFN alpha is secreted by leucocytes and IFN gamma is secreted by fibroblasts after viral infection.

10 1. Interferon gamma is an interferon elaborated by T lymphocytes in response to either specific antigen or mitogenic stimulation.

15 2. Interferon alpha includes a number of different subtypes that are elaborated by leukocytes in response to viral infection or stimulation with double-stranded RNA. IFN-alpha-2A and -2B are protein products made by recombinant DNA techniques and are used as antineoplastic agents. Interferon-alpha is one of the type I interferons (interferon type I) produced by peripheral blood leukocytes or lymphoblastoid cells when exposed to live or inactivated virus, double-stranded RNA, or bacterial products. It is the major interferon produced by virus-induced leukocyte cultures and, in addition to its pronounced antiviral activity, causes activation of natural killer cells.

20 3. Interferon alfa-2a is a type I interferon consisting of 165 amino acid residues with lysine in position 23. This protein is produced by recombinant DNA technology and resembles interferon secreted by leukocytes. It is used extensively as an antiviral or antineoplastic agent.

25 30 4. Interferon alfa-2b is type I interferon consisting of 165 amino acid residues with arginine in position 23. This protein is produced by recombinant DNA technology and resembles interferon secreted by leukocytes. It is used extensively as an antiviral or antineoplastic agent.

5. Interferon beta is an interferon elaborated by fibroblasts in response to the same stimuli as interferon alpha. Interferon-beta is one of the type I interferons produced by fibroblasts in response to stimulation by live or inactivated virus or by double-stranded RNA. It is a cytokine with antiviral, antiproliferative, and immunomodulating activity.

6. Interferon-b2 (interleukin-6) is a cytokine that stimulates the growth and differentiation of human B-cells and is also a growth factor for hybridomas and plasmacytomas. It is produced by many different cells including T-cells, monocytes, and fibroblasts. INF-b2 is a single chain 25 kD cytokine originally described as a pre B-cell growth factor, now known to have effects on a number of other cells including T-cells, which are also stimulated to proliferate. INF-b2 is an inducer of acute phase proteins and a colony stimulating factor acting on mouse bone marrow.

7. Interferon gamma is elaborated by T lymphocytes in response to either specific antigen or mitogenic stimulation.

ii. Tumor necrosis factor (TNF) is a tumor-inhibiting factor present in the blood of animals exposed to bacterial lipopolysaccharide. TNF preferentially kills tumor cells in vivo and in vitro, causes necrosis of certain transplanted tumors in mice, and inhibits experimental metastases. Human TNF alpha is a protein of 157 amino acids and has a wide range of pro-inflammatory actions. TNF may be present in the same concentrations as interleukins. Alternatively, TNF may be present in an effective concentration. An effective concentration of TNF is an concentration at which blood cells are activated by the present protocol.

C. Cell Stimulating Factors. Activating blood cells in the presence of one or more cell stimulation factors may be efficacious in alleviating aplastic anemia in the context of the present invention. Cell stimulating factors are contemplated to include such substances as granulocyte colony-stimulating factor granulocyte macrophage-colony stimulating factor and macrophage-

colony stimulating factor. Cell stimulating factors may be present in concentrations between about 10 and 50,000 IU/ml, between about 10 and 10,000 IU/ml, or between about 10 and 1000 IU/ml. Alternatively, an effective concentration of cell stimulating factors may be present. An effective concentration of cell stimulating factors is any concentration at which blood cells are activated by the present protocol.

1. Granulocyte colony-stimulating factor (G-CSF): G-CSF are glycoproteins synthesized by a variety of cells and are involved in growth and differentiation of hematopoietic stem cells. In addition, these factors stimulate the end-cell functional activity of stem cells.
2. Granulocyte-macrophage colony-stimulating factor (GM-CSF): GM-CSF is an acidic glycoprotein of 23 kD with internal disulfide bonds. GM-CSF is produced in response to a number of inflammatory mediators by mesenchymal cells present in the hemopoietic environment and at peripheral sites of inflammation. GM-CSF stimulates the production of neutrophilic granulocytes, macrophages, and mixed granulocyte-macrophage colonies from bone marrow cells and can stimulate the formation of eosinophil colonies from fetal liver progenitor cells.
3. Macrophage-colony stimulating factor (M-CSF): M-CSF is a cytokine synthesised by mesenchymal cells that stimulates pluripotent stem cells of bone marrow into differentiating towards the production of monocytes (mononuclear phagocytes). The compound stimulates the survival, proliferation, and differentiation of hematopoietic cells of the monocyte-macrophage series. It is a disulfide-bonded glycoprotein dimer with a mw of 70 kD and binds to a single class of high affinity receptor which is identical to the product of the c-fms proto-oncogene.
2. Ionophores. Ionophores are calcium or other cation specific reagents (such as polypeptides) which can traverse a lipid bilayer and a lipid soluble. There are two classes of ionophores: carriers and channel formers. Carriers, like valinomycin,

form cage-like structures around specific ions, diffusing freely through the hydrophobic regions of the bilayer. Channel formers, like gramicidin, form continuous aqueous pores through the bilayer, allowing ions to diffuse therethrough. In addition to the foregoing, suitable ionophores for the present protocol may include A23187 (calcimycin), ionomycin, geldanamycin, monensin (Na-salt), nystatin, polymyxin-B sulfate, and rapamycin. It is believed that carriers, such as A23187, accumulate calcium cations in response to pH gradients. A23187 possesses a dissociating carboxylic acid group and catalyzes an electrically neutral exchange of protons for other cations across the membrane (Hyono et al., BBA 389, 34-46 (1985); Kolber and Haynes, Biophysics Journal, 36, 369-391 (1981); Hunt and Jones, Biosci. Rep., 2, 921-928 (1982)). Two molecules of A23187 are present as carboxylate anions, and are thus available to carry to protons, or equivalents, back across the membrane after releasing the transported divalent cation. If present, ionophores may be present in concentrations between about 1 and 10,000 ng/ml, between about 1 and 1000 ng/ml, or between about 10 and 500 ng/ml. Alternately, ionophores may be present in an effective concentration. An effective concentration of ionophores is any concentration at which blood cells are activated, but not overactivated, by the present protocol. Excessive concentrations of activating agents may not be effective in the treatment approaches described herein.

Use, Packaging and Distribution

The delivery of activated cells can provide a statistically significant improvement in clinical parameters of a patient. For example, the administration of cell activated as described herein can result in a statistically significant increase in white blood cell counts, red blood cell counts hemoglobin levels and platelet counts. In general, continuation of the treatment procedure as described herein can result in a return to normal blood levels. In some embodiments, after four treatments, the patient can have an increase in each of white blood cell counts, red blood cell counts and hemoglobin of at least about 20%, in other embodiments at least about 35% and in other embodiments at least about 50%. Similarly, in some embodiments, platelet counts can increase by at least about 25%, in other embodiments at least about 50%, and in further embodiments at least about 100%. A person of ordinary skill in the art will recognize that additional ranges of blood parameter improvement within the explicit ranges presented are contemplated and are

within the present disclosure.

The activation compounds, such as one or more cytokines and/or one or more ionophore, can be mixed with an appropriate cell culture medium or a portion thereof for distribution. In alternative embodiments, one or more activation compounds can be packaged along with a cell culture medium or portions thereof for shipping. Similarly, a desired combination of activation compounds, such as one or more cytokines and one or more ionophores, can be packaged together for shipping, either mixed or in separate compartments. In any of these embodiments, the medium and/or activation compounds can be combined with any remaining medium components and/or activation compounds to form the desired medium for culturing cells under conditions to activate the cells. Also, in any of these embodiments, the compositions that are packaged together can include, for example, instructions for completing the cell culture medium with activation properties and/or instructions for performing the cell culturing.

The cell culturing can be performed at the facility that is treating the patient or the cell culturing to activate the cells can be performed at a remote location. In either case, the activated cells can be administered after a short period of time after harvesting from the cell culture to ensure that the cells remain viable. Alternatively, the cells can be stored under conditions that maintain the cells in a viable condition. For example, the cells can be stored at liquid nitrogen temperatures with a cryoprotectant. The cells can be prepared, for example using known procedures, at appropriate times for administration to the patient. For example, the cells can be suspended in a buffered saline solution for administration to the patient. Other known carriers, for example, can be used for delivery of the cells.

25

EXAMPLES

Example 1 - Treatment of Aplastic Anemia

I. Patients

Eight patients with verified histories of from one to six years of occupational exposure to benzene were subjected to the present regimen after their consents were obtained. The makeup of the patients was one male and seven females and the ages of the patients ranged from 24 to 41. All patients experienced symptoms of weakness, dizziness, fainting, and accelerated heart rates. Among these patients, four were hospitalized due to acute symptoms with bleeding. The hospitalized patients required blood or platelet transfusions. The other four patients experienced chronic symptoms and were treated with

standard therapies for four, six and 15 months, respectively. Bone marrow biopsies and aspiration samples were obtained from all patients to confirm hematopoiesis. Toxic levels of benzene were present in the blood and bone marrow of all patients.

5 II. Purification of Peripheral Blood Mononuclear Cells and Cell Culture

Peripheral blood mononuclear cells (PBMCs) were separated from patient blood samples (40-50 ml) by Ficoll-Hypaque centrifugation. The separated PBMCs were then placed in an appropriate volume (based on cell concentration) of RPMI 1640 with 10% fetal bovine serum under sterile conditions and cultured at 2×10^6 cells/ml for 48 hours in
10 the presence of interleukin-2 (IL-2) at 500 IU/ml (Chiron, Emeryville, CA), granulocyte macrophage-colony-stimulating factor (GM-CSF) at 200 IU/ml (Immunex, Seattle, WA), and calcium ionophore A23187 at 100 ng/ml (Sigma, St. Louis, MO). At the end of the culture period, adherent cells were scraped off the plastic surfaces of the culture vessels and harvested together with non-adherent cells. To harvest the cells, the cells were spun
15 down to form a cell pellet. Different numbers of cells were obtained for different patients. The harvested cells were washed twice in saline solution and administered to the patients. After washing, the cells were resuspended in 5 to 10 mls of saline, with the volume determined by the number of cells. These suspensions were further diluted with 50 ml of saline before administering the cells to the patients.

20

Treatment Protocol

Activated allogeneic PBMCs were used for a single patient (HC) in the first three treatments because the patient had experienced low blood counts, severe bleeding and infection. For the other patients, activated PBMCs were intravenously administered with
25 50 ml saline to the patients. The treatment was repeated every week for at least four weeks. The number of cells administered to a particular patient depended on the number of cells obtained from the patient.

III. Results

30 Hematological Parameters

Hematological parameters, white blood cell counts, red blood cell counts, hemoglobin levels, and platelet counts, were monitored before and after the treatment for each patient and are shown in Table 1. Data from these patients indicated that the therapy

was effective in enhancing the peripheral blood cell counts. Six patients experienced improvement of more than one subset listed and two patients had better platelet counts. The blood cell counts began to improve in most patients after two treatments and continued to improve throughout the time the present activated cells were administered.

5 Seven of the eight patients improved to the extent that some of their hemological parameters reach normal levels or levels approaching normal after completion of four treatments. Although blood cell counts of the patients improved from the therapy in general, improvements were not uniformly achieved. Some patients experienced limited improvement in red blood cell counts, but dramatic improvement in platelet counts. It was

10 noted that all patients' platelet counts were significantly increased.

Patient HC experienced more severe acute symptoms than the other patients. Additionally, patient HC had a bleeding problem as well. Because of the low yields of peripheral blood cells from patient HC, allogeneic PBMCs were used to stimulate patient HC's hematopoiesis. After three treatments using allogeneic cells, patient HC's blood

15 counts began to improve. After the three treatments of allogeneic cells, autologous PBMCs were then used to continue the therapy. Although patient HC's hemotological parameters were not corrected to normal levels after six treatments, patient HC continued to improve.

Discomfort due administering the present immunotherapy was mild to moderate.

20 Five patients experienced no appreciable discomfort. Three patients experienced chilling, fevers between 37 and 39 degrees C, headaches, nausea, vomiting, and loss of appetite after cell infusion. However, these symptoms were transient, typically lasting one to two days. Aspirin was administered when patients experienced discomfort.

25 Bone Marrow Hematopoiesis

Bone marrow biopsies and aspiration samples were obtained from all patients before the therapy began and two weeks after the final treatment. As shown in Figure 1, the histology of the bone marrow samples from three patients with the most severe samples indicated severe damage before the therapy was begun. After the therapy was

30 administered, remarkable improvements in bone marrow histology were found. With respect to patient HC, however, the improvement observed in patient HC's bone marrow was not coupled with improved peripheral blood counts.

Blood Transfusion

Before beginning treatment, four of the eight patients experienced severe symptoms, coupled with bleeding. These four patients required periodic transfusions of whole blood or platelets before and during the therapy. After four treatments, however, none of the patients experienced bleeding and whole blood and platelet transfusions were not continued.

Duration

The beneficial effects of the present cell-based therapy do not appear to be transient. All patients continued to have improved or stable hematological parameters after the therapy was discontinued. Some female patients experienced unstable blood counts during menstrual periods, but no patients experienced a relapse. Patient LC, who responded to the therapy, has experienced stable symptoms for more than two months since the final treatment (Figure 2).

Discussion

The results of this study suggest that administering activated PBMCs to patients with aplastic anemia is highly effective.

Some patients had close to normal bone marrow histologically, but had peripheral hematological parameters which were not as close to normal. To this end, it seemed that a time gap occurred between histological recovery of bone marrow and recovery of peripheral blood cell counts. Patients experiencing this gap were closely monitored and the patients' hematological parameters showed continued improvement. These patients sometimes took a few weeks or months to attain normal peripheral blood cell counts.

In analyzing the data generated by the study, it was noted that, among different compartments of the blood, increase in platelets was most evident, significant and rapid in patients benefiting from therapy. The initial increase in platelet counts was possibly due to the fact that platelets have a faster generation and differentiation interval. Other cell types of blood such as neutrophils, granulocytes and reticulocytes were also improved in agreement with the four parameters listed (data not shown). Platelet counts are likely more susceptible to benzene toxicity than other blood cells, but are the most responsive to the present therapy due to their faster generation interval.

Acquired aplastic anemia is a difficult disease to cure. However, the present immunosuppression therapy was very effective in treating this disease, for which bone

marrow transplants are the only known cure heretofore. However, in spite of the success of bone marrow transplants, this therapy has serious complications, e.g., tumors, (Socie et al., Malignant tumors occurring after treatment of aplastic anemia, N. Eng. J. Med. 1993; 329(16): 1152-1157) and graft-versus-host disease (Ferrara et al., Graft-versus-host disease, N. Engl. J. Med. 1991(324); 324: 667-674). Moreover, many patients cannot obtain bone marrow transplants due to the expense of the procedure and/or the lack of compatible donors. To this end, a simple and effective therapy with fewer side effects is needed to treat aplastic anemia. The results of this study indicate that aplastic anemia can be effectively treated with minimal side effects. The present cell-based immunotherapy is believed to be applicable to other types of anemia and bone marrow disorders as well. These disorders include those experienced by HIV (human immunodeficiency virus)-infected patients after cocktail chemotherapy and cancer patients with bone marrow failure after chemotherapy and radiotherapy, inherited aplastic anemia, and idiopathic thrombocytopenic purpura.

While not wishing to be bound by a specific theoretical basis for the operation of this invention, it is presently believed that several phenomena may be responsible for the favorable responses of patients to the present immunotherapy. A first theory is that the activated cells secrete multiple (perhaps partially unknown) effective factors simultaneously. These multiple factors, when working in concert, may have a synergistic combined effect. A second factor hypothesized for the effectiveness of the present therapy is that some presently unknown key factors for hematopoiesis are produced by activated immune cells. These unknown factors may be responsible, at least in part, for the effectiveness of the present therapy. A third factor which might be involved is that immune cells are capable of traveling to bone marrow and of delivering cytokines to hematopoietic stem cells and to other precursor cells at close range. Moreover, the present activated immune cells may be able to remain in close proximity to the marrow for periods sufficient to effect microenvironment improvement in the bone marrow. A fourth factor which might be responsible for the effectiveness of the present therapy is that cell contact between immune cells and hematopoietic cells may be essential for hematopoietic cell growth and differentiation. A fifth factor might be that activated immune cells, even in small amounts, may contribute to prevent the immune system from adversely influencing hematopoiesis. Quantities of PCMBs from 10-100 ml of blood are relatively small. However, these small quantities exerted large effects on bone marrow histology and hematopoiesis.

The results of administering blood cells activated by the present protocol are unexpected in view of results from previous studies. With the exception of one study, Young et al. (note 2) found administered growth factors (granulocyte-colony stimulating factor and granulocyte macrophage-colony stimulating factor) to affect neutrophil numbers only. The one study showed marked increases of neutrophil and platelet counts when granulocyte-colony stimulating factor was administered. Interleukin-3, administered alone or in combination with granulocyte macrophage stimulating factor had even less effect on myelopoiesis than the growth factors administered alone. Similarly (Liu et al., Cellular interactions in hemopoiesis, Blood Cells 1987; 13: 101-110 and Ettinghausen et al., Hematologic effects if immunotherapy with lymphokine-activated killer cells and recombinant interleukin-2 in cancer patients, Blood 1987; 69(6): 1654-1660), found that administering activated peripheral blood mononuclear cells and interleukin-2 to patients "emphasized" anemia and eosinophilia in patients receiving this therapy.

The present invention is also contemplated to include items of manufacture, which include separately packaged containers of one or more cytokine(s) and ionophore(s) as more fully described above. The container contents may be used to culture, and thereby activate, blood cells for use in the present therapeutic protocol. Instructions, such as on a label, may be present in the item of manufacture. A medium suitable for culturing blood cells may further be included.

Example 2 - Treatment of Platelet Deficiency

This example described the treatment of a 1 year five month old female patient with idiopathic thrombocytopenic purpura. The patient was diagnosed with the disease at about 9 months.

The patient was first treated with conventional therapy of corticosteroids and intravenous infusions of immunoglobulin. Although the patient responded to the conventional treatment, the patient became completely dependent on the corticosteroid therapy. To maintain sufficient platelet levels, the patient had to receive increasingly higher doses of corticosteroids.

Then, the patient was treated with an activated cell based therapy as described herein. The treatment was the same as described in Example 1 except that only 20 mls of blood was drawn from the patient each time, rather than 40-50 mls. The patient was treated once a week for 9 weeks. Ex vivo activated cells were administered on day 1, day 8, day 15, day 22, day 29, day 35, day 42, day 49 and day 56. At the same time that

immunotherapy with activated cells was initiated, corticosteroids and any other aspect of conventional therapy were completely withdrawn. The patient's platelet levels gradually improved during the treatment with activated cells as shown in Fig. 2. The patient had a lung infection at day 49 that correlated with a significant decrease in platelet number.

5 After the patient recovered from the infection, the patient's platelet numbers went back to normal levels.

All publications, patents, patent applications, and other documents cited herein are hereby incorporated by reference in their entirety. In the case of conflict, the present specification shall prevail.

10 Because numerous modifications of this invention may be made without departing from the spirit thereof, the scope of the invention is not to be limited to the embodiments illustrated and described. Rather, the scope of the invention is to be determined by the appended claims and their equivalents.

Table 1. Hematological Profiles of Patients Before and After Cell-Based Immune Therapy.

Patients	Age/Sex	No. of Treatment	Disease Type	WBC ($\times 10^3/\text{mL}$)	RBC ($\times 10^6/\text{mL}$)	HGB (g/dl)	PLT ($\times 10^3/\text{mm}^3$)	WBC ($\times 10^3/\text{mL}$)	RBC ($\times 10^6/\text{mL}$)	HGB (g/dl)	PLT ($\times 10^3/\text{mm}^3$)
HC	32/F	6	Acute	2.7 \pm 0.2	1.2 \pm 0.1	4.0 \pm 0.3	28 \pm 3	3.8 \pm 0.25	2.0 \pm 0.2	7.0 \pm 0.3	43 \pm 3
YM	29/F	4	Chronic	3.6 \pm 0.3	3.8 \pm 0.5	11.1 \pm 0.4	99 \pm 11	6.7 \pm 0.3	3.9 \pm 0.4	12.7 \pm 0.5	182 \pm 17
TB	33/F	4	Acute	3.2 \pm 0.2	2.3 \pm 0.3	8.4 \pm 0.4	47 \pm 5	4.7 \pm 0.3	3.2 \pm 0.3	11.5 \pm 0.7	107 \pm 11
LC	25/M	4	Acute	1.4 \pm 0.2	1.6 \pm 0.3	5.7 \pm 0.3	16 \pm 7	5.7 \pm 0.2	4.8 \pm 0.3	13.5 \pm 0.7	135 \pm 14
YX	25/F	4	Chronic	2.5 \pm 0.3	2.7 \pm 0.2	8.2 \pm 0.4	22 \pm 3	3.5 \pm 0.3	2.8 \pm 0.2	9.8 \pm 0.5	100 \pm 10
JX	29/F	5	Acute	2.7 \pm 0.7	3.0 \pm 0.2	8.1 \pm 0.3	44 \pm 18	4.5 \pm 0.7	3.6 \pm 0.1	12.0 \pm 0.4	126 \pm 8
ZL	41/F	4	Chronic	3.1 \pm 0.6	4.0 \pm 0.6	12.6 \pm 1.9	154 \pm 35	3.7 \pm 0.1	4.3 \pm 0.02	13.2 \pm 1.3	187 \pm 21
SC	29/F	4	Chronic	2.4 \pm 0.3	2.6 \pm 0.1	10.0 \pm 3.5	54 \pm 4	2.8 \pm 0.2	2.9 \pm 0.2	11.0 \pm 0.3	71 \pm 8

The hematological parameters were measured and analyzed for five consecutive days before and after the therapy. Data are expressed as means \pm standard deviation. WBC indicates white blood cells; RBC indicates red blood cells; HGB indicates hemoglobin; PLT indicates platelets.

WHAT IS CLAIMED IS:

1. A method of culturing blood cells, comprising culturing the blood cells in the presence of a cytokine and an ionophore.
- 5 2. The method of claim 1, in which the blood cells are cultured in the presence of effective amounts of the cytokine and the ionophore.
3. The method of claim 1, in which the blood cells are cultured in a medium comprising mammalian serum.
- 10 4. The method of claim 1, in which the blood cells are cultured for a period between about 10 and 90 hours.
5. The method of claim 1, in which the blood cells are cultured at a temperature between
15 about 30 and 42 degrees C.
6. The method of claim 1, in which the blood cells are cultured in the presence of interleukin-2 and granulocyte macrophage-colony stimulating factor.
- 20 7. The method of claim 1, in which the blood cells are allogeneic to a patient.
8. The method of claim 1, in which the blood cells are from an immunologically acceptable owner.
- 25 9. The method of claim 1, in which the blood cells are autologous to a patient.
10. The method of claim 1, in which the blood cells are cultured in the presence of A23187.
- 30 11. The method of claim 1, in which the blood cells are at least partially separated from sera before being cultured.
12. The method of claim 1, in which the blood cells are at least partially separated from sera by being centrifuged before being cultured.

13. A process of treating a patient with blood deficiencies, comprising administering ex vivo cultured blood cells to the patient to increase concentrations of blood components.
14. The process of claim 13, in which a therapeutically effective amount of blood cells
5 are administered to the patient.
15. The process of claim 13, in which the blood cells are cultured in the presence of a cytokine and an ionophore.
- 10 16. The process of claim 13, in which the blood cells are cultured in the presence of a cytokine and an ionophore comprising A23187.
17. The process of claim 13, in which the blood cells are cultured in the presence of a cytokine comprising interleukin-2.
- 15 18. The process of claim 13, in which the blood cells are cultured in the presence of a cytokine comprising macrophage-colony stimulating factor.
19. The process of claim 13, in which a therapeutically effective amount of blood cells is
20 administered to the patient.
20. The process of claim 13, in which the blood cells are autologous to the patient.
21. The process of claim 13, in which the blood cells are allogeneic to the patient.
- 25 22. The process of claim 13, in which the blood cells are cultured in the presence of a mammalian serum.
23. The process of claim 13, in which the blood cells are cultured in the presence of fetal
30 bovine serum.
24. The process of claim 13, in which the blood cells are from an immunologically acceptable donor.

25. A quantity of activated blood cells effective to treat blood deficiencies following injection into a patient.

26. The quantity of blood cells of claim 25, in which the quantity of blood cells is
5 cultured in the presence of a cytokine and an ionophore.

27. The quantity of blood cells of claim 26, in which the quantity of blood cells is cultured in the presence of effective concentrations of the cytokine and ionophore.

10 28. The quantity of blood cells of claim 25, in which a therapeutically effective amount of the cultured blood cells elevates peripheral blood cell populations in the patients when administered to the patients.

29. The quantity of blood cells of claim 25, in which the quantity of blood cells is
15 cultured in the presence of an interleukin and an ionophore.

30. The quantity of blood cells of claim 25, in which the quantity of blood cells is cultured in the presence of interleukin-2, macrophage-colony stimulating factor, and a calcium ionophore.
20

31. The quantity of blood cells of claim 25, in which the quantity of blood cells is cultured in the presence of interleukin-2, granulocyte macrophage-colony stimulating factor, and A23187.
25

1/2



Fig. 1

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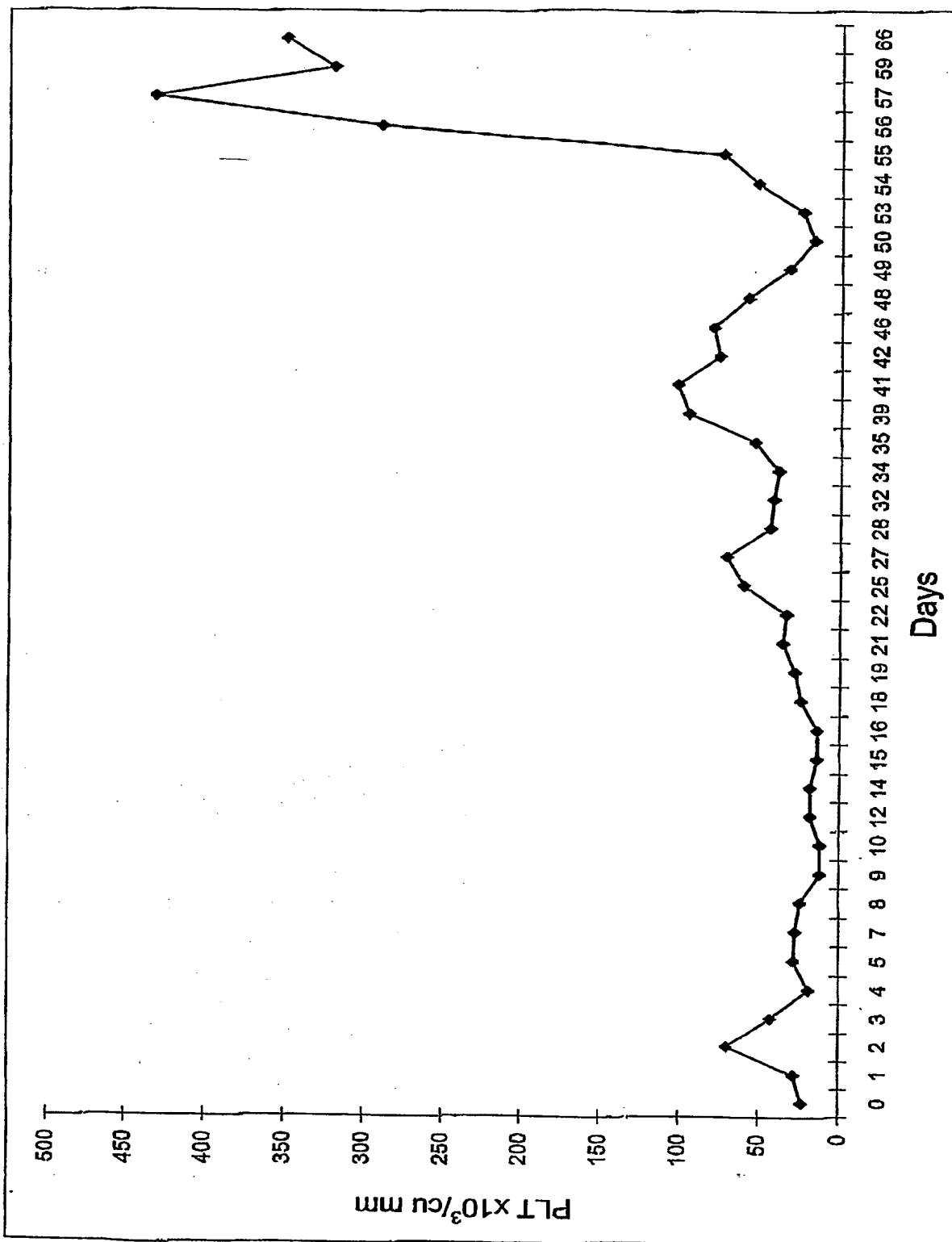


Fig. 2

Note: This international search report was established in addition to the report duly established by the competent International Searching Authority specified by the applicant. It is published for information only and has no legal status for the purposes of the PCT procedure (for example, in the computation of time limits).

INTERNATIONAL SEARCH REPORT		Application No PCT/US 03/14336
A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N5/06 A61K35/14		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N A61P C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, MEDLINE, PAJ, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 643 786 A (COHEN PETER A ET AL) 1 July 1997 (1997-07-01)	1-31
Y	The whole document, see e.g. claims 5 and 6 the whole document	1-31
X	WO 98 06823 A (BAXTER INT) 19 February 1998 (1998-02-19) the whole document	1-31
X	WO 01 34788 A (YOREH BIOTECHNOLOGIES LTD ; JEHUDA COHEN TAMAR (IL)) 17 May 2001 (2001-05-17) the whole document	1-31
	-/-	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search 30 July 2003		Date of mailing of the international search report 12/08/2003
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer Valcarcel, R

INTERNATIONAL SEARCH REPORT

Application No

PCT/US 03/14336

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X A	WO 01 62092 A (UNIV JEFFERSON) 30 August 2001 (2001-08-30) the whole document	13,14,25 1-12, 15-24, 26-31
X	ROROS J G ET AL: "CALCIUM IONOPHORE AND CYTOKINE TREATMENT OF HUMAN PERIPHERAL BLOOD MYELOID CELLS PRODUCES DENDRITIC CELLS WITH AN ENHANCED ABILITY TO SENSITIZE AUTOLOGOUS CD8+ T CELLS TO TUMOR ANTIGENS IN A SINGLE CULTURE STIMULATION" PROCEEDINGS OF THE ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, NEW YORK, NY, US, vol. 38, 12 April 1997 (1997-04-12), page 631 XP001018920 ISSN: 0197-016X the whole document	1-31
Y	the whole document	1-31
X	BEDROSIAN ISABELLE ET AL: "Granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-12 synergize with calcium ionophore to enhance dendritic cell function." JOURNAL OF IMMUNOTHERAPY, vol. 23, no. 3, May 2000 (2000-05), pages 311-320, XP009014769 the whole document	1-31
X	KOSKI GARY K ET AL: "Calcium ionophore-treated myeloid cells acquire many dendritic cell characteristics independent of prior differentiation state, transformation status, or sensitivity to biologic agents." BLOOD, vol. 94, no. 4, 15 August 1999 (1999-08-15), pages 1359-1371, XP002249511 ISSN: 0006-4971 the whole document	1-31

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INTERNATIONAL SEARCH REPORT

Application No

PCT/US 03/14336

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CZERNIECKI B J ET AL: "Calcium ionophore-treated peripheral blood monocytes and dendritic cells rapidly display characteristics of activated dendritic cells." JOURNAL OF IMMUNOLOGY (BALTIMORE, MD.: 1950) UNITED STATES 15 OCT 1997, vol. 159, no. 8, 15 October 1997 (1997-10-15), pages 3823-3837, XP001153936 ISSN: 0022-1767 the whole document</p>	1-31
X	<p>WESTERS THERESIA M ET AL: "A23187/IL-4 cultured leukemic dendritic cells stimulate autologous T cell-mediated apoptosis of acute myeloid leukemic blasts." BLOOD, vol. 98, no. 11 Part 1, 16 November 2001 (2001-11-16), page 121a XP009014739 43rd Annual Meeting of the American Society of Hematology, Part 1; Orlando, Florida, USA; December 07-11, 2001, November 16, 2001 ISSN: 0006-4971 the whole document</p>	1-31
X	<p>FARIES MARK B ET AL: "Calcium signaling inhibits interleukin-12 production and activates CD83+ dendritic cells that induce Th2 cell development." BLOOD, vol. 98, no. 8, 15 October 2001 (2001-10-15), pages 2489-2497, XP002249512 ISSN: 0006-4971 the whole document</p>	1-5, 7-16, 18-29
Y	<p>COOLEY M A ET AL: "CYTOKINE ACTIVITY AFTER HUMAN BONE MARROW TRANSPLANTATION III. DEFECT IN IL2 PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS IS NOT CORRECTED BY STIMULATION WITH CALCIUM IONOPHORE PLUS PHORBOL ESTER" BRITISH JOURNAL OF HAEMATOLOGY, vol. 73, no. 3, 1989, pages 341-347, XP009014779 ISSN: 0007-1048 the whole document</p>	1-31
A	<p>WO 95 20649 A (CELLCOR INC) 3 August 1995 (1995-08-03) the whole document</p>	1-31
-/--		

INTERNATIONAL SEARCH REPORT

Application No
PCT/US 03/14336

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 968 513 A (BRYANT JOSEPH ET AL) 19 October 1999 (1999-10-19) the whole document -----	13-31
A	EP 1 077 254 A (ZEN BIO INC) 21 February 2001 (2001-02-21) the whole document -----	13-31

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

international application No.
PCT/US 03/14336

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 13-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

Application No
PCT/US 03/14336

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			WO	9623060 A1	01-08-1996
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			US	6010905 A	04-01-2000
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			WO	9520649 A1	03-08-1995
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US 5968513	A	19-10-1999	AU	3792497 A	14-01-1998
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			EP	1077254 A2	21-02-2001
			JP	2001103963 A	17-04-2001
			US	6555374 B1	29-04-2003

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/14336

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 47/00; C12Q 1/00; C12N 5/00

US CL : 424/93.1, 93.7; 435/4,325,375,377,383,395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.7; 435/4,325,375,377,383,395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,010,905 A (COHEN et al) 04 January 2000 (04.01.2000), col. 5, 15-19, col 32-33 (Example 22).	1-31
X	BEDROSIAN et al. Granulocyte-Macrophage Colony-Stimulating Factor, Interleukin-2, and Interleukin-12 Synergize With Calcium Ionophore to Enhance Dendritic Cell Function. J. Immunotherapy. May-June 2000, Vol. 23, No. 3, pages 311-320, especially pages 312-313, 317.	1-12 and 25-31
A	CHOPRA et al. Interleukin 2, Interleukin 2 Receptor, and Interferon-gamma Synthesis and mRNA Expression in Phorbol Myristate Acetate and Calcium Ionophore A23187-Stimulated T Cell From Elderly Humans. Clin. Immunol. Immunopathol. November 1989, Vol. 53, pages 297-308, see entire document.	1-31
A	NOBREGA et al. Naturally Activated and Resting T Cells Differ in Their Activation Requirements For Growth and Secretory Activities. Cell Immunol. January 1990, Vol. 125, No.1, pages 120-129.	1-31
A	RODRIGUEZ et al. Regulation of Apoptosis in Interleukin-3-dependent hemopoietic Cells by Interleukin-3 and Calcium. HEMBO J., 1990, Vol. 9, pages 2997-3002.	1-31



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:		"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"I"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 June 2003 (27.06.2003)

Date of mailing of the international search report

05 NOV 2003

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

PCT/US03/14336

Continuation of B. FIELDS SEARCHED Item 3:

EAST, DIALOG, MEDLINE

search terms: inventor's name, ionophore, cytokine, blood, hematopoiesis, cell

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